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Hippocampal Neurogenesis and Cortical Cellular Plasticity in Wahlberg's Epauletted Fruit Bat: A Qualitative and Quantitative Study

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Key Words

Epomophorus wahlbergi · Hippocampus · Immunohistochemistry · Olfactory bulb · Optical fractionator · Piriform cortex · Plasticity

Abstract

Species-specific characteristics of neuronal plasticity emerging from comparative studies can address the functional relevance of hippocampal or cortical plasticity in the light of ecological adaptation and evolutionary history of a given species. Here, we present a quantitative and qualitative analysis of neurogenesis in young and adult free-living Wahlberg's epauletted fruit bats. Using the markers for proliferating cell nuclear antigen (PCNA), bromodeoxyuridine (BrdU), doublecortin (DCX) and polysialic acid neural cell adhesion molecule (PSA-NCAM), our findings in the hippocampus, olfactory bulb and cortical regions are described and compared to reports in other mammals. Expressed as a percentage of the total number of granule cells, PCNA- and BrdU-positive cells accounted for 0.04 in young to 0.01% in adult animals; DCX-positive cells for 0.05 (young) to 0.01% (adult); PSA-NCAM-positive cells for 0.1 (young) to 0.02% (adult), and pyknotic cells for 0.007 (young) to 0.005% (adult). The numbers were comparable to other long-lived, late-maturing mammals such as primates. A significant increase in the total granule cell number from young to adult animals demonstrated the successful formation and integration of new cells. In adulthood, granule cell number appeared stable and was surprisingly low in comparison to other species. Observations in the olfactory bulb and rostral migratory stream were qualitatively similar to descriptions in other species. In the ventral horn of the lateral ventricle, we noted prominent expression of DCX and PSA-NCAM forming a temporal migratory stream targeting the piriform cortex, possibly reflecting the importance of olfaction to these species. Low, but persistent hippocampal neurogenesis in non-echolocating fruit bats contrasted the findings in echolocating microbats, in which hippocampal neurogenesis was largely absent. Together with the observed intense cortical plasticity in the olfactory system of fruit bats we suggest a differential influence of sensory modalities on hippocampal and cortical plasticity in this mammalian order.

Introduction

Adult neurogenesis has been reported across several species living under laboratory conditions or in their natural habitats [Amrein et al., 2008]. Similarities and differences have been described in proliferative zones and rates of neuron formation [Kaslin et al., 2008] with species-specific differences attributed to lifespan and age [Kornack and Rakic, 1999; Lindsey and Tropepe, 2006; Ben Abdallah et al., 2010]. The functional relevance of adult-born

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neurons is still under intensive investigation, and studying natural populations with a wide range of ecological and behavioral demands is an advocated approach in the functional investigation of adult neurogenesis [Nottebohm, 2002; Treves et al., 2008; Barnea, 2009, 2010].

Bats occupy a unique ecological niche among mammals, and initial investigations in echolocating microbats (Microchiroptera) [Amrein et al., 2007] have demonstrated neurogenesis in the olfactory bulb and subventricular zone common also to rodents [Lois et al., 1996] and primates [Pencea et al., 2001]. However, hippocampal neurogenesis in microbats was observed to be largely absent and, when present, occurred at a low rate [Amrein et al., 2007]. Microbats and the non-echolocating fruit bats (Megachiroptera) share an evolutionary history with a unique adaptation to flight and longevity [Teeling et al., 2000; Shen et al., 2010; Wilkinson and South, 2002]. Fruit bats use olfactory and visual senses for spatial orientation and navigation [Hodgkison et al., 2007; Raghuram et al., 2009] and indeed, brain regions processing visual and olfactory information are proportionally larger in fruit bats compared to insectivore bats [Barton et al., 1995; Hutcheon et al., 2002]. Most interesting is that nectar- and fruit-eating lifestyles evolved independently in microbats and fruit bats [Thies et al., 1998; Hutcheon et al., 2002; Hodgkison et al., 2007], leading to convergent clustering of brain proportions in species occupying similar niches [de Winter and Oxnard, 2001]. Nectar- and fruit-eating microbats lack adult hippocampal neurogenesis [Amrein et al., 2007], and investigating fruit bats can therefore reveal if cellular plasticity in these species is subservient to phylogenetic constraints characterizing the Chiroptera or, if present, is a trait reflecting a mosaic organization caused by selective adaptation, as proposed by de Winter and Oxnard [2001].

In addition to neurogenesis in the hippocampus and olfactory bulb, cellular plasticity in the piriform cortex appears common to several species, perhaps indicative of the shared importance of olfaction [Nacher et al., 2001; Bernier et al., 2002; Nacher et al., 2002; Shapiro et al., 2007; Xiong et al., 2008; Luzzati et al., 2009]. The piriform cortex in its role of olfactory discrimination and autoassociative learning [Barnes et al., 2008; Linster et al., 2009] displays a high degree of synaptic plasticity reflected in the persistent expression of proteins associated with cellular plasticity as well. Given that fruit bats rely on precise olfactory discrimination, we therefore expect a high demand for plasticity in the piriform cortex.

Additionally, fruit bats are relatively long-lived (~20 years) [Wilkinson and South, 2002] and mature late; Wahlberg's epauletted fruit bat achieves sexual maturity at 15 months of age [Acharya, 1992]. Fruit bats therefore provide the opportunity to test the hypothesis that long-lived and late-maturing species have lower rates of adult hippocampal neurogenesis than short-living species such as rodents [Kornack and Rakic, 1999; Amrein and Lipp, 2009].

We present here a qualitative and quantitative assessment of neurogenesis in young and adult, free-living Wahlberg's epauletted fruit bats. For this, we used the following antibodies against proliferating cells and immature neurons: the endogenous marker proliferating cell nuclear antigen (PCNA) is a cofactor of DNA polymerases that is required for DNA replication [Bravo et al., 1987] and it is therefore expressed in proliferating cells, including mitotic neurons in the dentate gyrus [Ino and Chiba, 2000]. Bromodeoxyuridine (BrdU) is an exogenous marker that is incorporated into the DNA of proliferating cells, substituting endogenous thymidine [Gratzner, 1982]. Doublecortin (DCX) is a microtubule-associated protein that is expressed in the processes of postmitotic neurons during periods of migration and differentiation [Matsuo et al., 1998; Gleeson et al., 1999]. Polysialic acid neural cell adhesion molecule (PSA-NCAM) is a cell surface glycoprotein that is involved in the extension process of neurites [Doherty et al., 1990] expressed in adult-born neurons in the dentate gyrus [Seki and Arai, 1991]. In addition, dying cells were identified by their morphological appearance and total granule cell number estimated using the optical fractionator method. Our findings are discussed in relation to the ecology and behavior of this species, and the rate of neurogenesis of fruit bats is compared with rodents and primates.

Materials and Method

Animals and Treatment

The research reported herein was performed under guidelines established by the National Museums of Kenya and University of Nairobi. Wahlberg's epauletted fruit bats (n = 17) were captured in Nairobi, Kenya, and licenses were granted by the National Museums of Kenya. The fruit bats were assigned to one of two age groups (young and adult; table 1) based on the following criteria: closure of the femoral and humeral epiphyseal plate, body weight, forearm length and sexual maturity indicated by evidence of lactation or pregnancy in females and testis size in males. Young fruit bats were flying and feeding independently of adult fruit bats.

Eight animals (4 young and 4 adults) were injected with a single dose (100 mg/kg i.p.) of BrdU (BD Biosciences, San Diego, Calif., USA) and perfused 12 h later.

Table 1. Animal and section characteristics

Age group	Gender n	Mean body weight, g (SD)	Mean brain weight, g (SD)	Cryosections mean (range)	Plastic sections mean (range)
Young[1]	2 M, 5 F	53.5 (11)	1.7 (0.2)	8.7 (7–10)	19.4 (18–21)
Young[2]	1 F	64.1	2.0	10	
Adult[1]	1 M, 6 F	85.6 (7)	1.9 (0.1)	8.8 (7–10)	19.7 (18–23)
Adult[2]	2 F	88.3 (6)	2.0 (0.02)	12, 11	

[1] Brains were analyzed immunohistochemically for PCNA, BrdU, DCX and PSA-NCAM. Glycol-methylacrylate-embedded tissue was used for estimation of total granule cells and pyknotic cell bodies.
[2] Brains were analyzed immunohistochemically for PCNA and BrdU only.

Histology

Tissue Preparation

Animals were deeply anesthetized 12 h after capture with sodium pentobarbital (Nembutal®, 50 mg/kg) and transcardially perfused first with heparinized 0.9% saline, followed by phosphate-buffered 0.6% sodium sulfide solution and then cold 4% paraformaldehyde in 0.1 M phosphate buffer with 15% picric acid. Brains were removed and postfixed overnight at 4°C and transferred to 30% sucrose for 24 h.

Immunohistochemistry

From frozen right hemispheres, 40-μm coronal, horizontal and sagittal sections were cut and collected in 12 series. For each antibody, sections were run in a single batch with mouse brain tissue as control. Antibody concentrations that gave the best signal/background ratio were determined by dilution series. Anti-PCNA rabbit polyclonal antibody (1:30,000; Delta Biolabs, Gilroy, Calif., USA), anti-BrdU rat monoclonal antibody (1:400; Harlan Sera-Laboratories, Indianapolis, Ind., USA), anti-DCX goat polyclonal antibody (1:1,000; Santa Cruz Biotechnology, Santa Cruz, Calif., USA) and anti-PSA-NCAM mouse monoclonal antibody (1:5,000; Chemicon, Temecula, Calif., USA) were used to demonstrate proliferation and neurogenesis. One series was used for each marker. Sections were rinsed in Tris- (TBS)/Triton-buffered saline (pH 7.4), and pretreated in 0.6% hydrogen peroxide. Epitope retrieval in citrate buffer (ChemMate; DAKO, Glostrup, Denmark) at 94°C was done for PCNA immunolabelling. In the same buffer, sections were subjected to two short microwave pulses for DCX labeling. Sections were pre-incubated in normal serum for 1 h at room temperature before overnight incubation with primary antibodies at 4°C. This was followed by rinsing with TBS (pH 7.4), incubation for 2 h at room temperature with biotinylated secondary antibodies [1:1,000, goat anti-rabbit IgG (PCNA); rabbit anti-goat IgG (DCX); 1:500, donkey anti-mouse (PSA-NCAM)], rinses in TBS, and incubation for 35 min with the avidin-biotin complex (ABC; Vectastain® Elite ABC Kit; Vector Laboratories, Burlingame, Calif., USA). Immunoreaction was detected using 3,3′-diaminobenzidine (DAB) staining. DCX- and PSA-NCAM-stained sections were counterstained with hematoxylin (51275; Fluka, Buchs, Switzerland).

For BrdU immunolabelling, sections were incubated overnight at 4°C with primary antibody following DNA denaturation [2-hour incubation in 50% formamide/2× SSC (0.3 M sodium chloride and 0.03 M sodium citrate) at 65°C], rinsed for 15 min in 2× SSC, incubated for 30 min in 2 N hydrochloric acid at 37°C and again rinsed for 10 min in 0.1 M boric acid (pH 8.5). Detection with DAB followed incubation with a secondary goat anti-rat IgG (1:300; Jackson ImmunoResearch Laboratories, West Grove, Pa., USA) and ABC as described above.

Nissl Staining. Left hemispheres were processed as described in detail by Gatome et al. [2010]. Briefly, tissue was dehydrated, infiltrated and embedded with glycol methylacrylate solution (Technovit 7100; Kulzer, Wehrheim, Germany). Twenty-micron horizontal sections were Nissl stained with Giemsa solution (Merck, Darmstadt, Germany).

Quantitative Analysis of Cell Numbers

Estimates of total granule cell numbers were made in every 12th glycol-methylacrylate-embedded Nissl-stained horizontal section. Using the StereoInvestigator® software (MicroBrightfield, Colchester, Vt., USA), total granule cell numbers were estimated using the optical fractionator method [West et al., 1991] with a 40× oil immersion objective (N.A. 1.3) and a counting frame of 15 × 15 μm, a disector height of 10 μm and x-/y-steps of 210 μm. Section thickness was measured at every 6th sampling site. Cell numbers were estimated using number-weighted section thickness [Dorph-Petersen et al., 2001]. The same sections were also used for counts of pyknotic cells [Amrein et al., 2004] in the subgranular layer (SGL). Dying cells were counted throughout the thickness of the sections, omitting cells in the top focal plane. Cell numbers were multiplied with the inverse of the section sampling fraction, e.g. 12. In every 12th immunostained section, PCNA- and BrdU-positive cells were counted in the SGL while DCX- and PSA-NCAM-positive cells were counted in both SGL and granular cell layer following the rules described above for pyknotic cells.

Qualitative Analysis of Staining Intensity

Analysis was performed in the same series of sections used for the quantitative analysis of hippocampal neurogenesis. Staining patterns of PCNA, BrdU, DCX and PSA-NCAM were assessed in the olfactory bulb, subventricular zone (SVZ), temporal migratory stream and piriform cortex and scored as absent (0), weak staining of few, scattered immunopositive cells (1; e.g. fig. 3i), moderate numbers of intensely immunopositive cells (2; e.g. fig. 3k), strong staining with single and clustered, intensely immunopositive cells

Table 2. Unilateral quantification of hippocampal cell numbers

	Young				Adult				Age differences p values
	n	cell numbers means ± SD	in % of GC	CE^2/CV^2	n	cell numbers means ± SD	in % of GC	CE^2/CV^2	
PCNA	8	322 ± 74	0.04[1]	0.26	9	87 ± 43	0.009[1]	0.34	<0.0001
BrdU	4	261 ± 79	0.04[2]	0.30	4	87 ± 70	0.01[2]	0.15	0.016
DCX	7	422 ± 613	0.05	0.03	7	72 ± 40	0.008	0.22	0.158
PSA-NCAM	4	1,188 ± 1,070	0.14	0.02	3	244 ± 288	0.02	0.03	0.196
Pyknotic cells	7	54 ± 24	0.007	0.33	7	44 ± 33	0.005	0.26	0.528
GC	7	819,674 ± 117,196		0.39	7	945,273 ± 94,363		1.57	0.047

GC = Granule cells.
[1] Numbers calculated for 7 young and 7 adults.
[2] Numbers calculated for 3 young and 2 adults.

(3; e.g. fig. 3d), or very intense staining with clusters and chains of heavily immunopositive cells (4; e.g. fig. 3c, h).

Statistics

Statistical analyses were done using PASW Statistics version 17.0.0 (SPSS, Chicago, Ill., USA). Age-dependent variations were tested with general linear model and correlations with Pearson's r. Coefficients of error (CE) for cell number estimates were calculated according Gundersen et al. [1999] using the conservative m = 0 approach [Slomianka and West, 2005]. CE measurements provide information of the variance in measurement within an individual introduced by the stereological counting procedure. The CE^2/CV^2 ratio (CV = coefficient of variance = standard deviation/mean cell counts) was calculated to test if variance resulting from the estimation procedures (CE) was a major contributor to group variance (CV) [Gundersen and Jensen, 1987]. As a thumb rule, CE^2/CV^2 should be <0.5, indicating that the variance introduced by the stereological counting procedure accounts for <50% of the group variance [Slomianka and West, 2005].

Image Processing

Images were captured at magnifications of 40–100× using a MBF CX9000 camera (MicroBrightField). Adjustments were made to the brightness and color of the images to restore the original optical clarity.

Results

Animal details and the number of brain sections analyzed are shown in table 1. Adult fruit bats had a higher body weight than young fruit bats ($p < 0.001$). Brain weight did not vary between the age groups.

Hippocampal Region

We noted that PCNA-, BrdU-, DCX- and PSA-NCAM-positive cells were mostly located in the infrapyramidal blade of the dentate gyrus. For all markers, few stained cells were also observed in the hilus and molecular layer of the dentate gyrus. Estimates of immunopositive cell numbers and their relative proportions as a percentage of the resident granule cell population are shown in table 2 for both age groups.

Proliferation

PCNA and BrdU labelling patterns were typical compared to mouse tissue (fig. 1a–d). The number of PCNA-positive cells was higher in young than in adult fruit bats ($p < 0.001$; fig. 1c, d, 2). The number of BrdU-positive cells in young was higher than in adult fruit bats ($p = 0.016$; fig. 1a, b, 2). The numbers of BrdU-positive cells correlated with PCNA-positive cells ($r = 0.83$, $p = 0.011$). In young fruit bats, proliferating cells (BrdU and PCNA) accounted for 0.039% of the total granule cell number. In adult fruit bats, this number decreased to 0.009%.

Neuronal Differentiation

The PSA-NCAM labeling pattern was typical compared to mouse tissue (fig. 1e, f). The DCX-positive cells in the hippocampus (fig. 1g, h) stained considerably weaker in comparison to PSA-NCAM. The age-dependent down-regulation in staining intensity was more pronounced for DCX than PSA-NCAM (fig. 1e–h). In both age groups, some DCX-positive cells showed atypical staining, with brownish-stained somata that had no obvious processes. The numbers of DCX- and PSA-NCAM-positive cells were not significantly different between the age groups (fig. 2). Overall, a strong correlation was noted between the numbers of DCX- and PSA-NCAM-positive cells ($r = 0.89$, $p = 0.003$). Both markers for young neurons

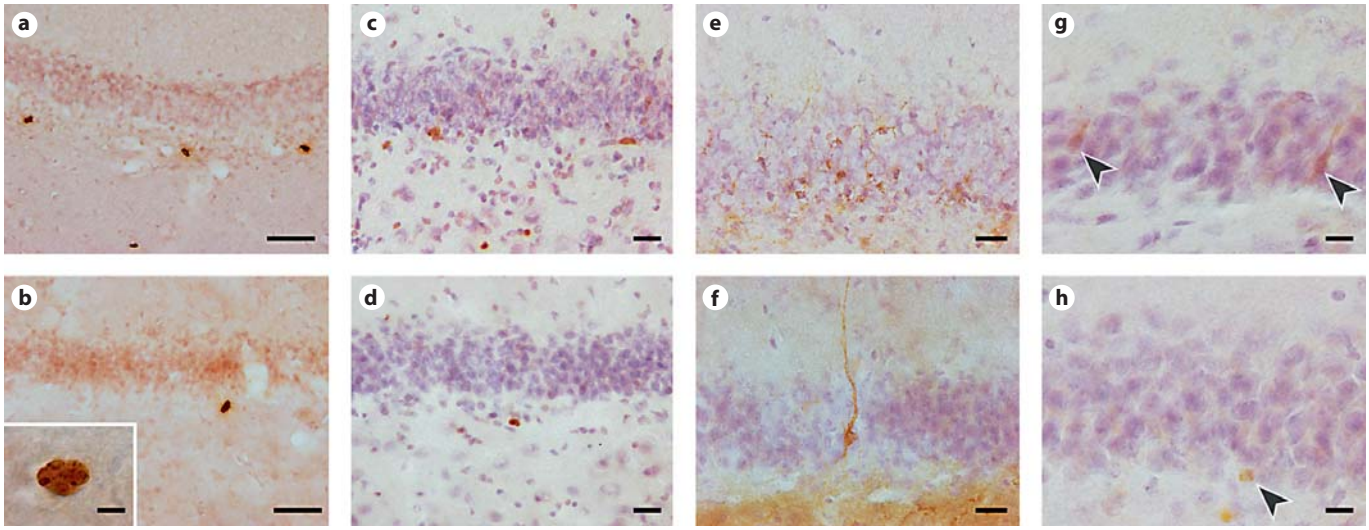

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Fig. 1. Proliferation and neurogenesis in the hippocampus of young (**a**, **c**, **e**, **g**) and adult (**b**, **d**, **f**, **h**) fruit bats. In young animals (**a**), more cells in the SGL of the dentate gyrus having incorporated BrdU are visible than in adults (**b**). PCNA-immunopositive cells in the SGL show a staining intensity similar to mouse tissue. PSA-NCAM- (**e**, **f**) and DCX- (**g**, **h**) immunopositive cells in the dentate gyrus stain considerably weaker than cortical and subventricular regions (see fig. 3) and in comparison to rodents. Scale bars: **a**, **b** 50 μm; **c**–**f** 20 μm; **g**, **h** 10 μm; inset in **b** 5 μm.

correlated with the number of proliferating, PCNA-positive cells (DCX-PCNA: r = 0.49, p = 0.045; PSA-NCAM-PCNA: r = 0.73, p = 0.039).

Pyknotic Cells

The age groups did not differ in the number of pyknotic cells (fig. 2). Pyknotic cell numbers were highly correlated to PCNA-positive cell numbers in adult (r = 0.909; p = 0.005) but not in young fruit bats (r = 0.229; p = 0.62). Pyknotic cells were also observed in the hilus and molecular layer of the dentate gyrus.

Granule Cell Number

Mean granule cell number in young fruit bats (819,674) accounted for 86% of the mean total number in adults (945,273; table 2). The granule cell number was higher in adult compared to young fruit bats (p = 0.047) Total granule cell number lay between mice (520,000) [Ben Abdallah et al., 2010] and rats (1,200,000) [West et al., 1991; Rapp and Gallagher, 1996].

CE^2/CV^2 Ratio

CE^2/CV^2 ratios (table 2) indicated that the variation in the data introduced through the methodology was a minor contributor to the observed group variances. The only exception was total granule cell number in adult fruit bats; however, in this case the average CE estimate was 11% only.

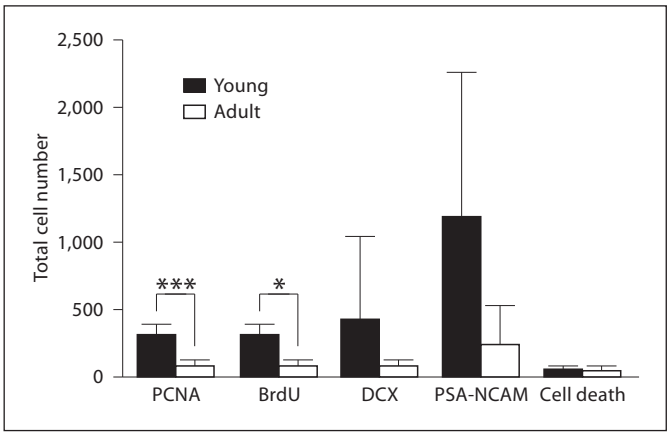

Fig. 2. Unilateral stereologically estimated cell numbers in the dentate gyrus of young and adult fruit bats differ only for PCNA ($p < 0.001$) and BrdU (p = 0.016), other cell numbers are not statistically different. Note the large individual variation in markers for young differentiating neurons (DCX and PSA-NCAM) in young animals. Bars = SD.

Olfactory Bulb and SVZ

Staining was typical to that observed in the mouse tissue. We observed immature neurons migrating to the olfactory bulb from the lateral ventricle through the rostral migratory stream, as shown in rodents and primates (fig. 3a–d) [Lois et al., 1996; Kornack and Rakic, 2001].

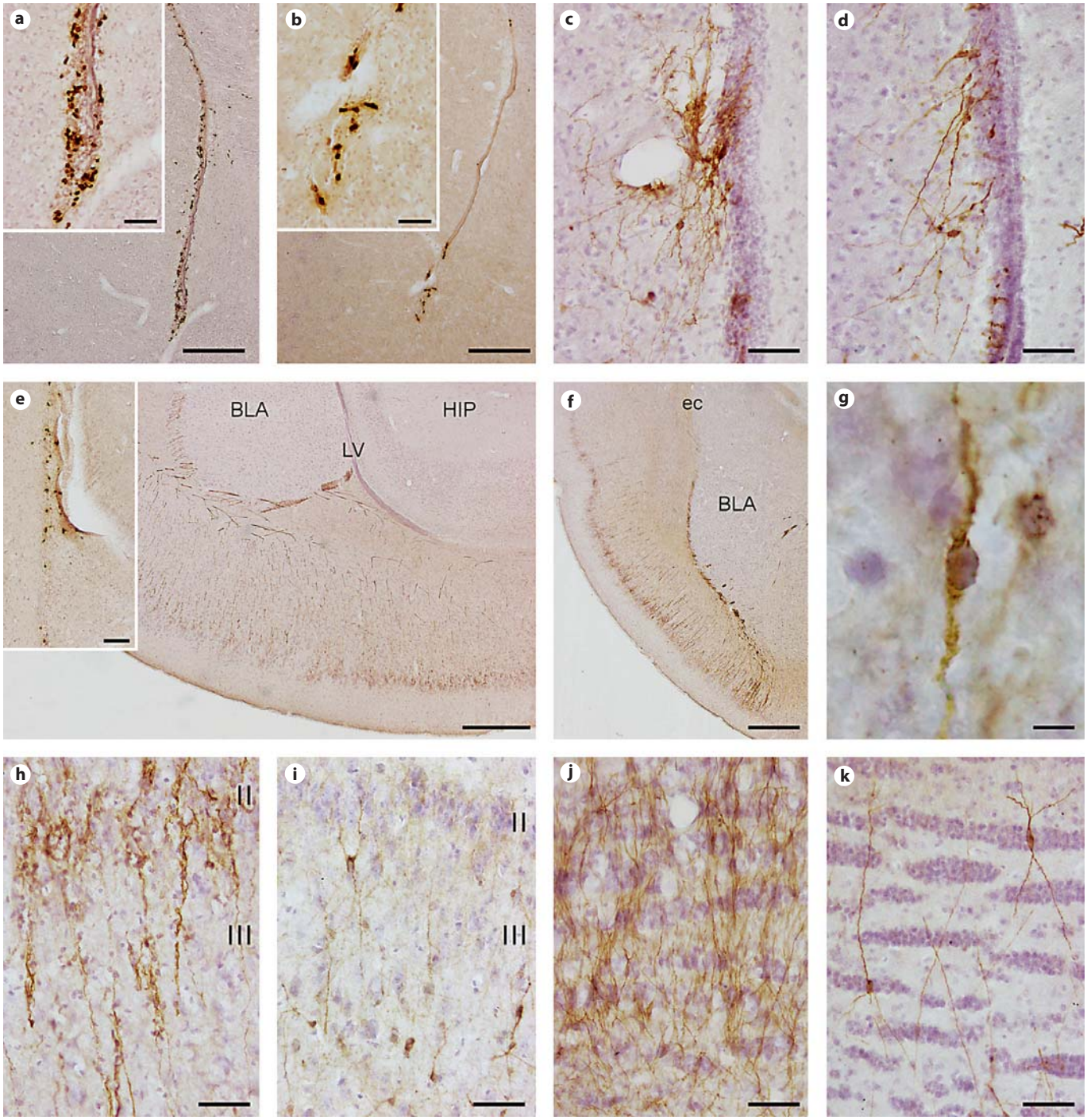

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Fig. 3. Proliferation and cellular neuronal plasticity in the SVZ, olfactory bulb, temporal migratory stream and piriform cortex in young and adult fruit bats. Proliferating cells visualized with BrdU in the SVZ decrease from young (**a**) to adult (**b**) fruit bats. The age-dependent down-regulation is obvious for DCX-positive cells in the SVZ, too (**c** young; **d** adult). Proliferating cells (BrdU, inset in **e**) in the temporal horn of the lateral ventricle are likely to be the founder population of migrating, PSA-NCAM-positive cells surrounding the amygdala and targeting the piriform cortex layer II: PSA-NCAM in sagital (**e**) and coronal (**f**) sections of a young fruit bat. In the piriform cortex, PSA-NCAM-positive cell bodies are located in layer II, abundant in young (**h**) and scarce in adult (**i**) animals. **g** Close-up of a PSA-NCAM-positive, radially migrating neuron in piriform layer II. In the olfactory bulb, DCX-positive cells forming extensive dendritic trees are abundant in young (**j**) and rare in adults (**k**). BLA = Basolateral amygdala; HIP = hippocampus; LV = lateral ventricle; ec = external capsule. Scale bars: **a**, **b**, **e**, **f** 500 μm; **c**, **d**, **h–k** and inserts in **a**, **b** 50 μm; **g** 5 mm; inset in **e** 100 μm.

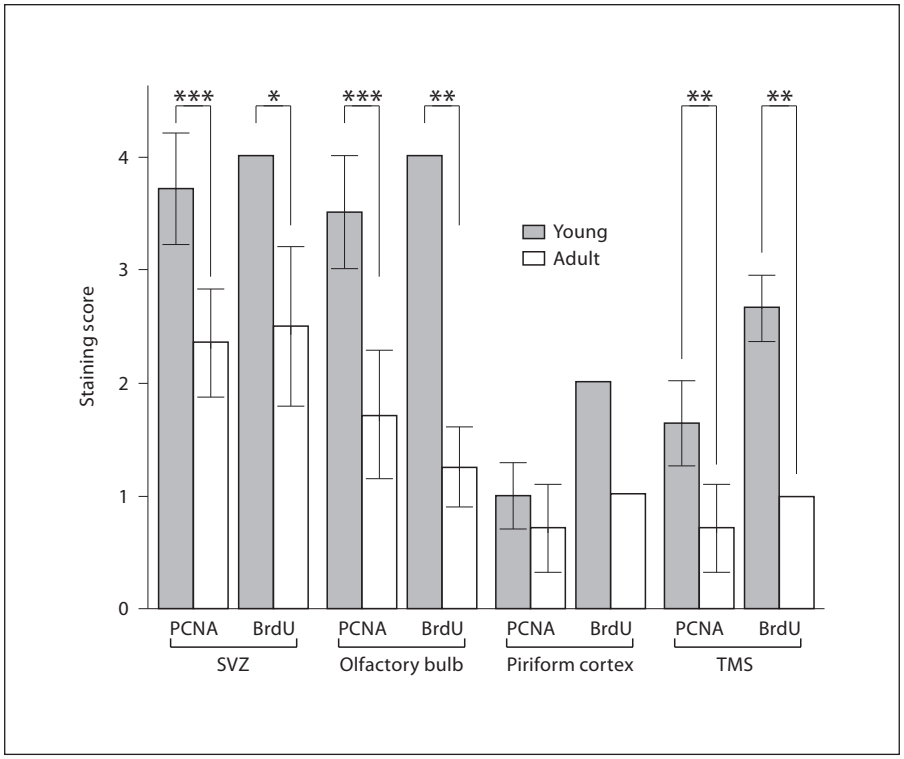

Fig. 4. Scored staining intensity for proliferation (PCNA and BrdU) in the SVZ (PCNA $p < 0.001$; BrdU $p = 0.028$), olfactory bulb (PCNA $p < 0.001$; BrdU $p = 0.001$), piriform cortex (PCNA $p = 0.147$) and temporal migratory stream (TMS: PCNA $p = 0.001$; BrdU $p = 0.004$) reveal an age-dependent down-regulation in all regions except in the piriform cortex. Score: 1 = weak; 2 = moderate; 3 = intense; 4 = very intense. Bars = SD.

There were individual clusters and chains of cells labeled for PCNA, BrdU, DCX and PSA-NCAM in the SVZ. DCX- and PSA-NCAM-positive cells displayed soma that extended stumpy dendritic processes resembling filopodia. In the olfactory bulb, proliferating cells appeared in small clusters in young fruit bats, and as individual cells in young and adult fruit bats (fig. 3j, k). The olfactory bulb showed lower reactivity compared to the SVZ. A continuation of the lateral ventricle was observed as a patent ventricle in the olfactory bulb of fruit bats. DCX- and PSA-NCAM-positive cells were noted in the bulbar ventricle wall and some spread radially towards the granule cells, extending fine processes. Scored staining intensities for PCNA and BrdU (fig. 4), and DCX and PSA-NCAM (fig. 5) showed that immunoreactivity decreased significantly with age (all measurements $p < 0.01$) except for a marginally non-significant decrease in PSA-NCAM in the olfactory bulb ($p = 0.055$).

Piriform Cortex

In young fruit bats, a temporal stream of DCX- and PSA-NCAM-positive cells was apparent from the ventral horn of the lateral ventricle and lined the external capsule in an apparent rostrodorsal direction towards the anterior piriform cortex (fig. 3e, f). More caudally, the migrating chain surrounded the amygdala targeting the posterior piriform cortex. At the caudal end, DCX- and PSA-NCAM-positive dendrites appeared perpendicular to the ventricle wall, ending in the posterior piriform cortex. The appearance of DCX- and PSA-NCAM-positive cells in the migratory stream resembled that described in the SVZ above. DCX- and PSA-NCAM-positive cells located in layers II and III were mostly oriented perpendicular to the pial surface (fig. 3h, i). Cell clustering was more obvious in the young fruit bats, and stained cells were of round, pyramidal and fusiform morphology. Most cells had several fine, elongated branching processes extending into the molecular layer above and white matter below. PCNA- and BrdU-positive cells along the ventricle walls were present in young animals, but scarce in adults (fig. 3, insert in e). Scored staining intensities for PCNA, BrdU, DCX and PSA-NCAM in the temporal migratory stream and in the piriform cortex were significantly higher in young than in adult fruit bats (all measurements $p < 0.01$) except for PCNA in the piriform cortex ($p = 0.15$; fig. 4, 5). Pyknotic cells were also observed in the piriform cortex layer II, being more frequent in young fruit bats.

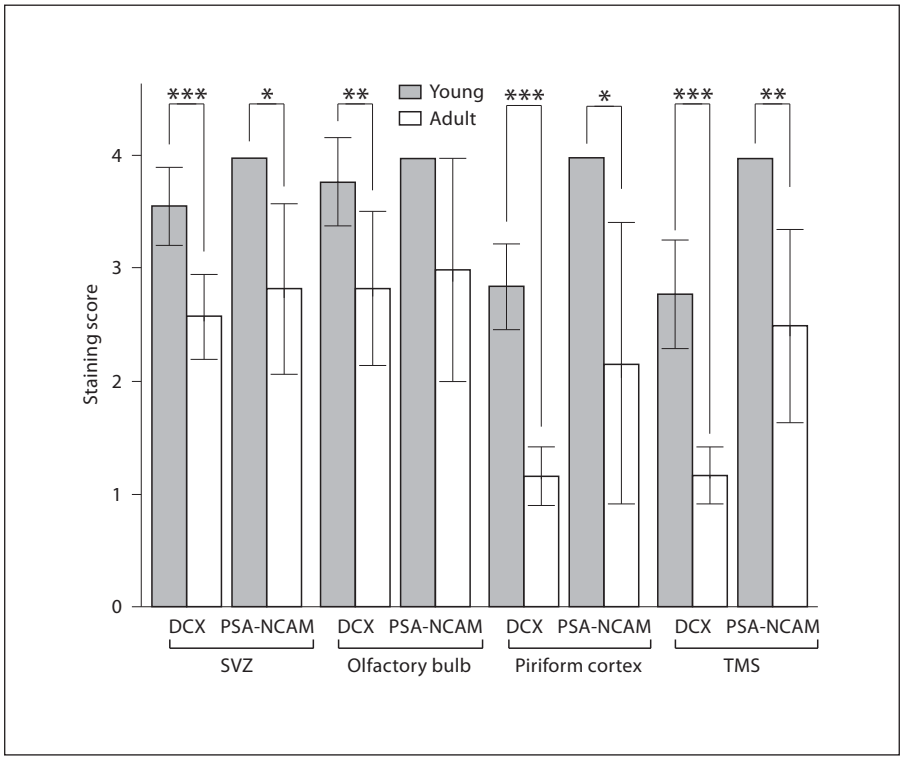

Fig. 5. Scored staining intensity for neuronal differentiation (DCX and PSA-NCAM) in the SVZ (DCX $p < 0.001$, PSA-NCAM $p = 0.011$), olfactory bulb (DCX $p = 0.009$, PSA-NCAM $p = 0.011$), piriform cortex (DCX $p < 0.001$, PSA-NCAM $p = 0.014$), and temporal migratory stream (TMS: (DCX $p < 0.001$, PSA-NCAM $p = 0.006$) reveal an age-dependent down-regulation in all regions except for the olfactory bulb (only PSA-NCAM). Score: 1 = weak; 2 = moderate; 3 = intense; 4 = very intense. Bars = SD.

Discussion

Quantitative data and a description of neurogenesis are provided for Wahlberg's epauletted fruit bat, a member of the non-echolocating, fruit- and nectar-feeding Megachiroptera. For this species, the extent of hippocampal neurogenesis was low, but markers for young neurons were predominant in other brain areas such as the piriform cortex. Our findings are discussed in relation to possible functional implications within the context of other mammalian species.

Hippocampal Neurogenesis

We applied two different markers for proliferating cells (PCNA and BrdU) and differentiating neuronal cells (DCX and PSA-NCAM) and observed good correlations between the markers, indicating that, within the limits of explanatory power of immunohistochemical techniques, proteins specific for proliferating cells and differentiating neurons are expressed in Wahlberg's epauletted fruit bat, as they are in rodents, albeit to a lower extent as described below.

Comparative Assessment

Normalizing the number of neurogenesis-related cell counts to the number of resident granule cells is necessary to assess the relative importance of the newly formed cells in relation to the functions subserved by the resident granule cells. Normalized estimates allow across-species comparisons and can provide valuable insights into biologically relevant species- or higher-order-specific regulatory mechanisms of adult neurogenesis. We therefore compared our normalized cell estimates in fruit bats to data published in rodents and primates which had been collected using design-based procedures, as applied in our study.

Proliferation

In 2-month-old C57BL/6 mice, proliferating, PCNA-positive cells [Snyder et al., 2009] amounted to ~1% of the total granule cell number [Ben Abdallah et al., 2010]. BrdU-positive cells in a 2-hour survival paradigm were ~0.6% in C57BL/6 [Mandyam et al., 2004; Ben Abdallah et al., 2010] and ~0.1% in rats [Pham et al., 2003]. In primates, BrdU-positive cells [Leuner et al., 2007] amounted to ~0.03% [Kozorovitskiy, 2005] in adult marmosets. In the fruit bat, the number for PCNA- and BrdU-positive cells ranged from 0.04 to 0.01% of the total granule cell number in young and adult animals, respectively. Thus, fruit bats showed a low level of proliferation in the SGL, being comparable to primates. Our observation of similar

numbers of BrdU- and PCNA-positive cells contrasts with the twofold higher number of PCNA-positive cells in comparison to BrdU-positive cell counts in mice [Mandyam et al., 2004; Snyder et al., 2009]. However, it fits well with others, reporting only minor differences in rodents [Gil et al., 2005; Olariu et al., 2007].

Neuronal Differentiation

With regard to neuronal differentiation, our findings fall below those of rodents indicating a low level of neurogenic activity in the dentate gyrus of fruit bats. In mice, DCX-positive cells decreased from ~2.1% in 2-month-old to 0.2% in 7- to 9-month-old animals [Ben Abdallah et al., 2010]. In adult Sprague-Dawley rats, the PSA-NCAM-positive cells amounted to 1.6% of total granule cells [Pham et al., 2003]. In fruit bats, PSA-NCAM-positive cells accounted only for 0.1 to 0.02% of the total numbers of granule cells in young and adult animals. The percentages of DCX-positive cells were even lower (0.05 to 0.01%, respectively). DCX and PSA-NCAM had an overlapping window of expression in rodents [Ming and Song, 2005; Lledo et al., 2006]. In fruit bats, the number of PSA-NCAM-positive cells in the dentate gyrus was twice as high as DCX-positive cells. Although staining intensity of DCX was in general weaker than PSA-NCAM, both markers overlapped in their regional staining and age-dependent down-regulation.

Cell Death

In fruit bats, the pyknotic cells amounted to 0.007 and 0.005% of the total granule cells in young and adult animals, respectively, again closer to the numbers in primates [Jabès et al., 2010] than in mice [Ben Abdallah et al., 2010]. In relation to the number of proliferating cells, far fewer dying cells were observed in young than in adult fruit bats. This agrees with similar observations in young and adult mice [Ben Abdallah et al., 2010].

Comparing the normalized data between species, we observed a clear trend that fruit bats share low adult hippocampal neurogenesis with long-lived primates. Common to rodents and primates, we found an aged-dependent down-regulation of neurogenic activity in the fruit bat hippocampus. Young fruit bats had significantly more BrdU- and PCNA-positive cells and, albeit not significant, higher numbers of young cells of the neuronal lineage than adults. The net increase in total granule cells at the transition from young to adult fruit bats supported the assumption of an experience-dependent addition of new cells [Tashiro et al., 2007], behaviorally corresponding with the exposure to increasingly novel sensory stimuli in early flight periods. In adult animals, cell proliferation and cell death are tightly linked in rodents [Amrein et al., 2004] and fruit bats, suggesting similarities in the regulation of adult hippocampal neurogenesis in mammals. Essentially, we observed an age-dependent decrease in proliferation, as shown in other mammals [Kuhn et al., 1996; Ben Abdallah et al., 2010].

The number of dentate gyrus granule cells is unlikely to change in adulthood due to the low variation (CV < 10%) in granule cell numbers in adult fruit bats, despite the large range of ages contributing to our animal sample. We did expect higher numbers of granule cells in fruit bats, because the hippocampal size index in a sister species of Wahlberg's epauletted fruit bat is by a factor of two larger than that of rats and corresponds well to that of primates [Gatome et al., 2010]. However, the mean number of granule cells in adult fruit bats of 950,000 is below the reported total granule cells of 1,200,000 in rats [West et al., 1991; Rapp and Gallagher, 1996]. Fruit bats have the largest hippocampus among the Chiroptera [Baron et al., 1996b; Hutcheon et al., 2002], and its functional relevance has been linked to the use of complex habitats and large territories [Safi and Dechmann, 2005], high demands for spatiotemporal memory processing in locating food sources [Hutcheon et al., 2002] and high alertness needed for species roosting in open space [Baron et al., 1996a]. The efficiency of hippocampal processing in Wahlberg's epauletted fruit bat is not mirrored in high numbers of resident or newly born granule cells, but might be linked to other cellular adjustments such as a highly differentiated entorhinal cortex [Gatome et al., 2010].

Olfactory System

In young fruit bats, we found a prominent rostral migratory stream leading to the olfactory bulb, as well as a temporal stream of young neurons originating from the ventral horn of the lateral ventricle targeting the piriform cortex. Inspection of coronal, horizontal and sagittal DCX- and PSA-NCAM-stained series of sections indicated that DCX- and PSA-NCAM-positive cells in anterior and posterior piriform cortex originated from the temporal migratory stream, as shown in rabbits [Luzzati et al., 2009] and primates [Bernier et al., 2002]. There is no indication of a caudoventral migratory stream targeting the anterior piriform cortex, as shown in rats [Shapiro et al., 2007].

The staining pattern of immunopositive cells in layer II of the piriform cortex with their dendrites extending in layer I and deep layers resembled closest to that reported in cats. Noteworthy, Cai et al. [2009] did not advocate

that these cells migrate from the ventricles into the cortical areas, stating that DCX-positive cells in the piriform cortex are resident. We observed an age-related decrease in DCX and PSA-NCAM expression in the piriform cortex of the bat, as described in the rat [Shapiro et al., 2007], with adult fruit bats displaying weak immunoreactivity in the temporal stream and piriform cortex.

Relationship between Sensory Modalities and Neuronal Plasticity?

Expression of DCX and PSA-NCAM in the piriform cortex appears to be a common feature across several species for whom olfaction is important. The olfactory bulb sends direct input to the piriform cortex where several forms of plasticity have been reported in response to odor experiences [Wilson et al., 2004]. Integration of new neurons, which in the olfactory bulb is necessary in readapting to ongoing environmental changes and to maintain maximal discrimination [Petreanu and Alvarez-Buylla, 2002; Rochefort et al., 2002], could represent an adaptive process in the piriform cortex, too. The flower-visiting and fruit-eating microbats did not express DCX in the piriform cortex [Amrein, unpubl. observations] possibly because they have not developed their olfactory system to the same extent, relying more on echolocation for food detection [Hutcheon et al., 2002]. Cellular plasticity in response to sensory processing requirements thus reflects a mosaic trait in brain organization, despite the shared ecological niche and the common evolutionary ancestry of the two bat groups.

The abilities of the microbats to discriminate between group members and perform spatial tasks are aided by auditory mechanisms [Mueller and Emlen, 1957; Mueller and Mueller, 1979; Boughman and Wilkinson, 1998], which modulate the hippocampus in a manner different from rodents [Ulanovsky and Moss, 2007]. Possibly, the adaptive value of the auditory system [O'Neill and Suga, 1979; Suga and O'Neill, 1979] takes precedence over other structures involved in spatial and olfactory tasks [Hutcheon et al., 2002]. Olfactory sensory stimulation may be linked to hippocampal neurogenesis as olfactory bulbectomy in rats leads to decreased hippocampal neurogenesis [Jaako-Movits and Zharkovsky, 2005; Pope and Wilson, 2007]. If foraging demands and sensory modalities influence the occurrence and nature of hippocampal plasticity in fruit bats (as well), it is worth undertaking an investigation in a species such as the Egyptian fruit bat. This fruit bat species faces similar foraging demands as Wahlberg's epauletted fruit bat and uses all three sensory modalities, olfaction, vision and echolocation. A further approach would be to investigate hippocampal neurogenesis in the straw-colored fruit bat, a closely related species which uses larger home ranges and shows migratory behavior.

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